Supplemental File

Report

The Trimethylamine-N-Oxide Generating Enzyme Flavin Monooxygenase 3 is a Central Regulator of Cholesterol Balance.

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Extended Experimental Procedures:

Mice, Diets, and Treatments

Antisense oligonucleotide (ASO)-mediated knockdown was accomplished using 20-mer phosphorothioate ASOs designed to contain 2'-0-methoxyethyl groups at positions 1 to 5 and 15 to 20. All ASOs used in this work were synthesized, screened, and purified as described previously (Crooke et al., 2005) by ISIS Pharmaceuticals, Inc. (Carlsbad, CA). Creation of NPC1L1-^{LiverTg} mice has been described previously (Temel et al., 2007), and we used the high overexpressing line (L1-Tg112) that had been backcrossed onto the C57BL/6N background for > 6 generations here. For microarray analysis in WT vs. NPC1L1-^{LiverTg}, female mice were fed a diet containing 0.2% cholesterol (wt/wt) along with receiving a control (non-targeting) ASO for 8 weeks in order to compare to subsequent studies. For microarray analysis in control vs. ACAT2 antisense oligonucleotide (ASO)-treated mice, we purchased female C57BL/6N mice (6-8 weeks old) from Harlan (Indianopolis, IN, USA), and then placed them on a semisynthetic low-fat, high-cholesterol diet (10% of energy as palm oil-enriched fat, 0.2% cholesterol w/w) and maintained on this diet for the remainder of the study. After 6 weeks of high-cholesterol diet feeding, mice were injected intraperitoneally biweekly with 25 mg/kg of either non-targeting control ASO (5'-TCCCATTTCAGGAGACCTGG-3') or and ASO directed against murine ACAT2 (5'-TTCGGAAATGTTGCACCTCC-3') as previously

described (Marshall et al., 2014). After one week of treatment, mice were sacrificed for subsequent microarray analysis. For FMO3 knockdown studies, young (6-8 week old) female C57BL/6 mice were purchased from Harlan (Indianopolis, IN, USA), and started on either a low (0.02%, wt/wt) or high (0.2%, wt/wt) cholesterol diet and injected intraperitoneally biweekly with 25 mg/kg of either non-targeting control ASO (5'-TCCCATTTCAGGAGACCTGG-3') or and ASO directed against murine FMO3 (5'-TGGAAGCATTTGCCTTTAAA-3') for 8 consecutive weeks. For LXR agonist studies, T0901317 was suspended in a vehicle containing 1.0% carboxymethylcellulose (CMC) and 0.1% Tween 80. Mice were gavaged with either vehicle or 25 mg/kg T0901317 once daily for a period of seven days as previously described (Temel et al., 2010). All mice were maintained in an American Association for Accreditation of Laboratory Animal Care-approved pathogen-free animal facility, and all experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic and Wake Forest University School of Medicine.

Adenoviral Overexpression Studies

For FMO3 overexpression studies control adenovirus or adenovirus driving expression murine FMO3 were prepared as previously described (Bennett et al., 2013) using the AdEasy system (Agilent). All viruses were purified by cesium chloride gradient ultracentrifugation. For *in vivo* overexpression, 10^9 plaque-forming units (PFUs) were injected into the tail vein of recipient mice. For FMO3 adenoviral-mediated overexpression studies male C57BL/6 mice were fed a low (0.02%, wt/wt) cholesterol diet for a total of 3 weeks. Following two weeks of dietary induction, mice received a tail vein injection of either a control adenovirus (pAdTrack-CMV) or an adenovirus driving overexpression of murine FMO3 (pAdTrack-CMV-mFMO3). Thereafter, plasma was collected 3 days post adenoviral administration, and tissues and gall bladder bile were collected 7 days post adenoviral administration. Feces were quantitatively collected during days 4-7 post adenoviral administration.

Antibiotic Treatment Studies

To examine the role of gut microbial metabolism in FMO3 ASO-mediated alteration in cholesterol balance and hepatic inflammation female C57BL/6 mice were started on a low (0.02%, wt/wt) cholesterol diet at 6-8 weeks of age and injected intraperitoneally with either a control ASO or FMO3 ASO (50 mg/kg body weight per week) for a total of 5

weeks. Following four weeks of dietary induction and ASO treatment, mice either continued to receive normal drinking water or were switched to water supplemented with a poorly absorbed antibiotic cocktail as previously described (Wang et al., 2011) for 7 consecutive days. After only 4 days of antibiotic treatment all mice were gavaged with 50 µl soybean oil containing trace amounts of ^{14}C-cholesterol and ^{3}H-sitosterol and place on wire bottoms for 3 additional days for quantitative fecal collection. After 7 days of antibiotic treatment mice were necropsied for tissue and blood collection.

Dietary TMAO Addback Studies

To determine whether adding back the FMO3 product TMAO could rescue FMO3 ASO-mediated alteration in cholesterol balance and hepatic inflammation female C57BL/6 mice were started on a low (0.02%, wt/wt) cholesterol diet at 6-8 weeks of age and injected intraperitoneally with either a control ASO or FMO3 ASO (50 mg/kg body weight per week) for a total of 5 weeks. Following four weeks of dietary induction and ASO treatment, mice either continued to receive the same (0.02%, wt/wt) cholesterol diet or were switched to a diet containing 0.02%, wt/wt cholesterol with supplemental TMAO (0.3% wt/wt) for 7 consecutive days. After only 4 days of diet switch all mice were gavaged with 50 µl soybean oil containing trace amounts of ^{14}C-cholesterol and ^{3}H-sitosterol and place on wire bottoms for 3 additional days for quantitative fecal collection. After 7 days of dietary TMAO addback mice were necropsied for tissue and blood collection.

Necropsy Conditions

To keep results consistent all experimental mice were fasted for 4 hours (from 9:00 a.m. to 1:00 p.m.) prior to necropsy. At necropsy, all mice were terminally anesthetized with ketamine/xylazine (100-160mg/kg ketamine-20-32mg/kg xylazine), and a midline laparotomy was performed. Blood was collected by heart puncture. Following blood collection, a whole body perfusion was conducted by puncturing the inferior vena cava and slowly delivering 10 ml of saline into the heart to remove blood from tissues. Tissues were collected and immediately snap frozen in liquid nitrogen for subsequent biochemical analyses.

Plasma Lipid and Lipoprotein Analyses

Plasma triacylglycerol levels were quantified enzymatically (L-Type TG M, Wako Diagnostics, Richmond, VA, USA). Total plasma cholesterol levels were quantified enzymatically (Pointe Scientific, Canton, MI, USA). The distribution of cholesterol across lipoprotein classes was performed by size exclusion chromatography (FPLC using a superose-6 column) coupled with an online enzymatic cholesterol quantification as previously described (Brown et al., 2008a; Brown et al., 2008b; Brown, et al., 2010).

Quantification of Plasma and Liver Trimethylamine and Trimethylamine-N-Oxide

Quantification of TMA and TMAO in mouse plasma was performed using stable isotope dilution HPLC with online electrospray ionization tandem mass spectrometry on an API 365 triple quadrupole mass spectrometer (Applied Biosystems, Foster, CA) with upgraded source (Ionics, Bolton, ON, Canada) interfaced with a Cohesive HPLC (Franklin, MA) equipped with phenyl column (4.6 × 250 mm, 5 μm Rexchrom Phenyl; Regis, Morton Grove, IL), and the separation was performed as reported previously (Wang et al., 2011). TMAO and TMA were monitored in positive MRM MS mode using characteristic precursor-product ion transitions: m/z 76 →58, and m/z 60→44, respectively. The internal standards TMAO-trimethyl-d9 (d9-TMAO) and TMA-d9 (d9-TMA) were added to plasma samples before sample processing and were similarly monitored in MRM mode at m/z 85→68, and m/z 69→49. Various concentrations of TMAO and TMA standards and a fixed amount of internal standards were spiked into control plasma to prepare the calibration curves for quantification of plasma TMAO and TMA. For d9-TMA and d9-TMAO quantification in FMO activity analyses, 1,1,2,2-d4 choline (Sigma) was used as an internal standard followed by a 0.5 ml 3K cutoff centrifugal filter (Millipore) of sample prior to LC-MS/MS analysis. The characteristic precursor-product ion transition for 1,1,2,2-d4 choline is m/z 108→60 monitored in positive MRM MS mode

Biliary Cannulations and Biliary Lipid Analyses

For biliary cannulation studies, mice were anesthetized with isofluane (4% induction, 2% maintenance), and common bile duct cannulations were performed to collect newly secreted bile for a 15-minute period (Temel et al., 2010; Brown et al., 2008b). Briefly, once fully anesthetized, the peritoneal cavity was opened to the rib cage, and the duodenum was exposed. Through an incision in the duodenum, a cannula (polyethylene

10 tubing; ID 0.28 mm; OD 0.61 mm) filled with sterile saline was inserted through the sphincter of Oddi into the common bile duct. The cannula was tied to the bile duct, and newly secreted bile was allowed to fill the cannula. Following successful surgery, the exposed viscera were wetted with saline and covered with a saline-soaked gauze pad. To maintain physiological body temperature, the mice were placed on a heating pad, and bile was collected for a brief 15-minute period. In separate cohort of mice, gall bladder bile was collected for comparison purposes. For analysis of biliary lipid concentrations, a measured volume (5–10 µl) of gall bladder or newly secreted (collected during common bile duct cannulation) was extracted with 2:1 chloroform/methanol in the presence of 10 µg 5α-cholestane. The organic phase was analyzed for cholesterol content by gas-liquid chromatography, and for PL content using Phospholipids C enzymatic assay kit (Wako Diagnostics, Richmond, VA, USA) using a detergent solubilization method (Temel et al., 2010; Brown et al., 2008b). The aqueous phase was analyzed for BA content using an enzymatic assay employing hydroxysteroid dehydrogenase (Turley and Dietschy, 1978).

Intestinal Cholesterol Absorption and Fecal Neutral Sterol Loss Measurements

Intestinal cholesterol absorption was measured using the dual fecal isotope assay, and fecal neutral sterol loss was measured by gas chromatography as previously described (Temel et al., 2005).

Bile Acid Pool Size and Composition

Pool size was determined as the total bile acid content of the small intestine, gallbladder, and liver as previously described (Koeth et al., 2013). Briefly, these tissues were pooled and extracted together in ethanol with nordeoxycholate (Steraloids) added as an internal standard. The extracts were filtered (Whatman paper #2), dried and resuspended in water. The samples were then passed through a C18 column (Sigma Catalog # 52615-U) and eluted with methanol. The eluted samples were again dried down and resuspended in methanol. A portion of this was subjected to HPLC using Waters Symmetry C18 column (4.6 × 250 mm No. WAT054275, Waters Corp., Milford, MA) and a mobile phase consisting of methanol: acetonitrile: water (53:23:24) with 30mM ammonium acetate, pH 4.91, at a flow rate of 0.7 ml/min. Bile acids were detected by evaporative light scattering detector (Alltech ELSD 800, nitrogen at 3 bar, drift tube temperature 40^0C) and identified by comparing their respective retention times to those

of valid standards (Taurocholate and Tauro-β-muricholate from Steraloids; Taurodeoxycholate and Taurochenodeoxycholate from Sigma; Tauroursodeoxycholate from Calbiochem). For quantitation, peak areas were integrated using software Chromperfect Spirit (Justice laboratory software) and bile acid pool size was expressed as μmol/100 g body weight (bw) after correcting for procedural losses with nor-deoxycholate.

Immunoblotting

Whole tissue homogenates were made from tissues in a modified RIPA buffer as previously described (Brown et al., 2004), and protein was quantified using the BCA assay (Pierce). Proteins were separated by 4–12% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and proteins were detected after incubation with specific antibodies as previously described (Brown et al., 2004; Brown et al., 2008a; Brown et al., 2008b; Brown et al., 2010). Antibodies used include: anti-FMO3 rabbit polyclonal (ABCAM #Ab126790), anti-β actin rabbit monoclonal (Cell Signaling Technologies #4970), anti-CHOP mouse monoclonal (Cell Signaling Technologies #2895), anti-Src rabbit monoclonal (Cell Signaling Technologies #2123), and anti-phospho-Src^{Tyr416} rabbit monoclonal (Cell Signaling Technologies # 6943).

Microarray and Quantitative Real-Time PCR Analysis of Hepatic Gene Expression

Tissue RNA extraction was performed as previously described for all mRNA analyses (Brown et al., 2008a; Brown et al., 2008b; Brown, et al., 2010; Lord et al., 2012). For microarray analyses, hepatic total RNA samples were further purified using RNeasy MinElute Cleanup Kit (Qiagen # 74204) followed by quality assessment using an Agilent 2100 bioanalyzer. Samples with RIN values > 8.0 were carried forward for cRNA synthesis and hybridization to GeneAtlas MG-430 PM Array Strips (Affymetrix, Santa Clara, CA) following the manufacturer's recommended protocol. Briefly, approximately 250 ng of purified total RNA was reverse transcribed and biotin labeled to produce biotinylated cRNA targets according to the standard Affymetrix GeneAtlas 3'-IVT Express labeling protocol (GeneAtlas 3' IVT Expression Kit User Manual, P/N 702833 Rev. 4, Affymetrix). Following fragmentation, 6 μg of biotinylated cRNA was hybridized for 16 hr at 45 °C on the Affymetrix GeneAtlas Mouse MG-430 PM Array Strip. Strips were washed and stained using the GeneAtlas Fluidics Station according to standard Affymetrix operating procedures (*GeneAtlas™ System User's Guide* (P/N 08-0306

Rev.A January 2010). Strips were subsequently scanned using the GeneAtlas Imager system according to the standard Affymetrix protocol. Fluidics Control, Scan control and data collection was performed using the GeneAtlas Instrument Control Software version 1.0.5.267. The raw data generated were normalized using the robust multi-array average (RMA) method (Irizarry et al., 2003), and functional annotation to gene ontology was performed using Ingenuity-IPA software (Ingenuity Systems, Inc., Redwood City, CA). All microarray analyses were performed by the Wake Forest School of Medicine Microarray Shared Resource Core. Quantitative real time PCR (Q-PCR) analyses were conducted as previously described (Brown et al., 2008a; Brown et al., 2008b; Brown, et al., 2010; Lord et al., 2011). mRNA expression levels were calculated based on the ΔΔ-CT method. Q-PCR was conducted using the Applied Biosystems 7500 Real-Time PCR System. Primers used for Q-PCR are available on request.

Hepatic Cholesterol, Triglyceride, and Phospholipid Analyses

Extraction of liver lipids and quantification of molecular species by either mass spectrometric or enzymatic methods was performed as previously described (Lord et al., 2011; Ivanova et al., 2007; Myers et al., 2011). Briefly, total cholesterol (TC), cholesteryl ester (CE), free cholesterol (FC), triglyceride (TG) mass was measured using enzymatic assays as described previously (Temel et al., 2010; Brown et al., 2008a; Brown et al., 2008b). For glycerophospholipid analyses, liver tissue was extracted using a modified Bligh and Dyer procedure (Bligh and Dyer 1959). Approximately 10 mg of frozen mouse liver was homogenized in 800 µl of ice-cold 0.1 N HCl:CH_3OH (1:1) using a tight-fit glass homogenizer (Kimble/Kontes Glass Co, Vineland, NJ) for about 1 min on ice. Suspension was then transferred to cold 1.5 ml Eppendorf tubes and vortexed with 400 µl of cold $CHCl_3$ for 1 min. The extraction proceeded with centrifugation (5 min, 4^{o}C, 18,000 x g) to separate the two phases. Lower organic layer was collected and solvent evaporated. The resulting lipid film was dissolved in 100 µl of isopropanol:hexane:100 mM NH_4COOH(aq) 58:40:2 (mobile phase A). Quantification of glycerophospholipids was achieved by the use of an LC-MS technique employing synthetic odd-carbon diacyl and lysophospholipid standards. Typically, 200 ng of each odd-carbon standard was added per 10-20 mg tissue. Glycerophospholipids were analyzed on an Applied Biosystems/MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a Shimadzu high pressure liquid chromatography system with a Phenomenex Luna Silica column (2 × 250

mm, 5-µm particle size) using a gradient elution as previously described (Ivanova et al., 2007; Myers et al. 2011). The identification of the individual species, achieved by LC-MS/MS, was based on their chromatographic and mass spectral characteristics. This analysis allows identification of the two fatty acid moieties but does not determine their position on the glycerol backbone (*sn-1* versus *sn-2*).

Quantification of Plasma and Liver Oxysterol Levels

22(R)-hydroxycholesterol, 25-hydroxycholesterol and 25(R),26-hydroxycholesterol ("27"-hydroxycholesterol) were purchased from Steraloids (Newport, RI, USA), 24(S)-hydroxycholesterol and 24(S)-25-epoxycholesterol were purchased from Biomol International (Plymouth Meeting, PA, USA) and the deuterated internal standard (IS) 25-hydroxycholesterol-D_6 was from CDN Isotopes (Point-Claire, Quebec, Canada). Pyridine was from Sigma-Aldrich (St. Louis, MO, USA), *N,O*- Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was from Pierce (Rockford, IL), and all other chemicals were from Carlo Erba Reagenti (Rodano, Milan, Italy). Oxysterols from mouse liver and plasma were extracted and purified as de-scribed (Lund and Diczfalusy 2003) using Strata Si-1 columns (500 mg/6 ml) (Phenomenex, Torrance, CA, USA) in the solid phase extraction step. Dried extracted samples were converted to trimethylsilyl ethers by adding 250 µl pyridine/BSTFA (1:1) and incubating for 30 minutes at 60°C. After drying under a nitrogen flow, samples were resuspended with 50 µl hexane and analyzed by GC-MS using an Agilent 6890/ 5973 system equipped with the same column and using the same conditions described (Lund and Diczfalusy 2003). Analyses were performed in selected ion monitoring (SIM) mode using the following target ions: *m/z* 173 for 22(R)-hydroxycholesterol, *m/z* 145 for 24(S)-hydroxycholesterol, *m/z* 131 for 25-hydroxycholesterol, *m/z* 129 for both 25(R),26-hydroxycholesterol and 24,25-epoxycholesterol, and *m/z* 137 for the IS (25-hydroxycholesterol-D_6). Different retention times in the column allowed separation of oxysterols with the same target ion.

J774 Cell Culture and [^{3}H]-Cholesterol Loading for Macrophage RCT Studies

J774 mouse macrophages were a generous gift from Dr. George Rothblat (The Children's Hospital of Philadelphia). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. [^{3}H]-Cholesterol loading was initiated by incubating cells for 48 hours with 5 µCi/ml [^{3}H]-cholesterol and 100 µg/ml acetylated LDL in 10% FBS containing RPMI-

1640. The resulting foam cells were washed twice with phosphate buffered saline and equilibrated for an additional 12-hour period in serum free RPMI-1640 supplemented with 0.2% bovine serum albumin (BSA). Cells were then harvested and resuspended in serum-free RPMI-1640 immediately before injection. An aliquot of cells was extracted using the method of Bligh and Dyer (1959), and lipids were separated by thin layer chromatography (TLC) using a 70:30:1 (hexane:diethyl ether:acetic acid) solvent system. Following TLC separation J774 foam cells were found to have ~62% [^{3}H]-cholesteryl ester and ~38% [^{3}H]-free cholesterol. On average, the cell suspension contained ~10x10^6 cells/ml at ~3x10^6 dpm/ml. All cell suspensions were analyzed microscopically in order to count and to ensure viability before injection, and all mice were injected within 5 minutes of resuspension of freshly isolated foam cells.

***In Vivo* Macrophage RCT Studies in FMO3 ASO-Treated Mice**

In vivo measurement of macrophage RCT was conducted essentially as we have described previously (Temel et al., 2010), with minor modifications. In studies described here, female C57BL/6 mice were fed a low (0.02%, wt/wt) cholesterol diet and treated with either a control (non-targeting) ASO or an ASO targeting knockdown of FMO3 for 6 weeks. During the last week of treatment, mice also were orally gavaged with either a vehicle or an exogenous LXR agonist (T0901317; 25 mg/kg once daily). 48 hours before necropsy, mice were injected intraperitoneally with ~500 μl [^{3}H]-cholesterol labeled foam cell suspension containing ~10x10^6 cells/ml at ~3x10^6 dpm/ml. To allow quantitative fecal collection, mice were housed individually on wire bottom cages for 48 hours with *ad libitum* access to food and water. At 24, and 48 hours post injection, blood was collected via the submandibular vein, and 20 μl of isolated plasma was used to determine [^{3}H]cholesterol recovery. The [^{3}H]cholesterol distribution across plasma lipoproteins was determined for the 48-hour time point following size exclusion chromatography of plasma as previously described (Temel et al., 2010). At 48 hours post macrophage injection, mice were anesthetized with ketamine/xylazine (100-160mg/kg ketamine-20-32mg/kg xylazine), and a midline laparotomy was performed for tissue and bile collection. To determine recovery of macrophage-derived [^{3}H]cholesterol as [^{3}H]cholesterol or [^{3}H]bile acids in each tissue compartment, we used the following tissue-specific extraction protocols. For bile samples, 10 μl of gall bladder bile was extracted by adding 1 ml diH_20 and 3 ml 2:1 chloroform:methanol and then vortexing. The phases were split by adding 2 ml chloroform, vortexing, and centrifuging at 1000 x g for 10 minutes. The bottom phase

containing the [^{3}H]-cholesterol was carefully removed into a 7 ml scintillation vial, and dried under a N_2 stream. The remaining top phase containing the [^{3}H]-bile acids was subsequently re-extracted with 3 additional volumes (5 ml) of chloroform to completely remove [^{3}H]-cholesterol. All chloroform phases were pooled in the same scintillation vial and dried under a N_2 stream. The remaining cholesterol-depleted top phase containing [^{3}H]-bile acids was then also transferred to a 7 ml scintillation vial and completely dried under N_2. All dried samples were resuspended in 5 ml of scintillation cocktail and subjected to liquid scintillation counting to determine [^{3}H] recovery. Biliary lipid mass was analyzed using enzymatic methods as previously described (Brown et al., 2007, Temel et a. 2007).

To simultaneously determine [^{3}H]cholesterol and [^{3}H]bile acid recovery and sterol mass in feces, feces were quantitatively collected for 48 hours and then dried in a vacuum oven at 70^{o}C overnight. Feces were ground into a fine powder, and a measured amount of powdered feces (~100 mg) was extracted as follows. All samples received 100 µg of 5-alpha-cholestane as an internal standard for subsequent sterol mass determinations. Thereafter, samples were saponified by adding 2 ml 95% ethanol and 200 µl 50% KOH (w/v in diH_2O) and then incubating for 3 hours at 70^{o}C with periodic vortexing. The saponified samples were extracted by adding 2 ml hexane and 2 ml diH_2O with vortexing after each addition. The samples were then centrifuged at 2700 rpm at room temperature for 10 minutes to split the phases. The upper hexane phase (containing [^{3}H]-cholesterol) was removed, and the remaining lower phase was re-extracted 3 times with 2 ml hexane. All [^{3}H]-cholesterol hexane phases were pooled, and completely dried down under N_2, in a 7 ml scintillation vial. A small aliquot of the remaining bottom phase (containing [^{3}H]-bile acids) was transferred to another 7 ml scintillation vial and completely dried under N_2. Dried samples were then resuspended in 5 ml of scintillation cocktail and subjected to scintillation counting for [^{3}H] recovery determination. Mass fecal neutral sterol analysis was performed by gas liquid chromatography (GLC) as previously described (Brown et al., 2008; Temel et al., 2007).

For tissue (i.e. liver & small intestine wall) [^{3}H]cholesterol and [^{3}H]bile acid recovery, a piece of the liver (~100 mg) or entire intestinal segments were extracted for a minimum of 24h at room temperature in 3.75 ml 2:1 chloroform:methanol with frequent vortexing. Following extraction, 1.25 ml of chloroform was added to each sample, and vigorously vortexed. Thereafter, 1.25 ml of diH_2O was added to each sample, followed by vigorous vortexing. The phases (top phase containing and [^{3}H]bile acid and bottom phase

containing and [^{3}H]cholesterol) were split by centrifuging samples at 1000 x g for 10 minutes. The bottom phase containing the [^{3}H]-cholesterol was carefully removed into a 7 ml scintillation vial, and dried under a N_2 stream. The remaining top phase containing the [^{3}H]-bile acids was subsequently re-extracted with 3 additional volumes (3 ml) of chloroform to completely remove [^{3}H]-cholesterol. All chloroform phases were pooled in the same scintillation vial and dried under a N_2 stream. The remaining cholesterol-depleted top phase containing [^{3}H]-bile acids was then also transferred to a 7 ml scintillation vial and completely dried under N_2. All dried samples were resuspended in 5 ml of scintillation cocktail and subjected to liquid scintillation counting to determine [^{3}H] recovery. Total tissue recovery is calculated per total organ weight, and all macrophage RCT data are expressed at the % of dose injected recovered in each respective tissue.

***In Vivo* Determination of Very Low Density Lipoprotein (VLDL) Secretion**

Female C57BL/6 mice were injected with control ASO or FMO3 ASO and maintained on a high cholesterol diet (0.2%; wt/wt) for a period of 6 weeks prior to experiment. After a 4 hour fast, mice were anesthetized with isoflurane (4% for induction, 2% for maintenance) and Triton WR 1339 (500 mg/kg body weight; Sigma) was delivered via retro-orbital injection, to block lipolysis. Thereafter, blood samples were collected from anesthetized mice by retro-orbital bleeding at 0, 0.5, 1, 2, and 3 h after injection. Plasma was harvested from the blood samples and used to quantify total cholesterol (TC), cholesteryl ester (CE), free cholesterol (FC), triglyceride (TG) mass using enzymatic assays as described previously (Temel et al., 2010; Brown et al., 2008a; Brown et al., 2008b).

Liver Histopathology

Formalin-fixed livers were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Lobular inflammation was scored in a blinded fashion by a board certified pathologist (Xiuli Liu – Cleveland Clinic).

Statistical Analysis

Data are expressed as the mean ± S.E.M., and were analyzed using either a one-way or two-way analysis of variance (ANOVA) followed by Student's t tests for post hoc analysis using JMP version 5.0.12 software (SAS Institute, Cary, NC). For microarray analysis we used empirical Bayes method implemented in R package limma (Smyth 2004).

Extended Experimental Procedures References:

Bennett, B.J., de Aguiar Vallim, T.Q., Wang, Z., Shih, D.M., Meng, Y., Gregory, J., Allayee, H., Lee, R., Graham, M., Crooke, R., et al. (2013) Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. Cell Metab. *17,* 49-60.

Bligh E.G., and Dyer W.J. (1959). A rapid method for total lipid extraction andpurification. Can. J. Biochem. Physiol. *37,* 911-917**.**

Brown, J.M., Boysen, M.S., Chung, S., Fabiyi, O., Morrison, R.F., and McIntosh, M.K. (2004). Conjugated linoleic acid induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. J. Biol. Chem. *279,* 26735-26747.

Brown, J.M. Betters, J.L., Lord, C., Ma, Y., Han, X., Yang, K., Alger, H.M., Melchior, J., Sawyer, J.K., Shah, R., et al. (2010) CGI-58 knockdown in mice causes hepatic steatosis but prevents diet-induced obesity and glucose intolerance. J. Lipid Res. *51,* 3306-3316.

Brown, J.M., Chung, S., Sawyer, J.K., Degirolamo, C., Alger, H.M., Nguyen, T., Zhu, X., Duong, M.N., Wibley, A.L., Shah, R., et al. (2008a) Inhibition of stearoyl-coenzyme A desaturase 1 dissociates insulin resistance and obesity from atherosclerosis. Circulation *118,* 1467-1475.

Brown, J.M., Bell, T.A. 3rd, Alger, H.M., Sawyer, J.K., Smith, T.L., Kelley, K., Shah, R., Wilson, M.D., Davis, M.A., Lee, R.G., et al. (2008b) Targeted depletion of hepatic ACAT2-driven cholesterol esterification reveals a non-biliary route for fecal sterol loss. J. Biol. Chem. *16,* 10522-10534.

Crooke, R.M., Graham, M.J., Lemonidis, K.M., Whipple, C.P., Koo, S., and Perera, R.J. (2005). An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. J. Lipid Res. *46,* 872-884.

Ivanova, P.T., Milne, S.B., Byrne, M.O., Xiang, Y., and Brown H.A. (2007). Glycerophospholipid identification and quantitation by electrospray ionization mass spectrometry. Meth. Enzymol. *432,* 21-57.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., and Speed, T.P. (2003). Exploration, normalization and summaries of high density oligonucleotide array probe level data. Biostatistics *4,* 249-264.

Lord, C.C., Betters, J.L., Ivanova, P.T., Milne, S.B., Myers, D.S., Madenspacher, J, Thomas, G., Chung, S., Liu, M., Davis, M.A. et al. (2012). CGI-58/ABHD5-derived signaling lipids regulate systemic inflammation and insulin action. Diabetes *61,* 355-363.

Lund, E.G., and Diczfalusy, U. (2003) Quantification of receptor ligands by mass spectrometry. Methods Enzymol. *364,* 24-37.

Marshall, S.M., Kelley, K.L., Davis, M.A., Wilson, M.D., Lee, R.G., Crooke, R.M., Graham, M.J., Rudel, L.L., Brown, J.M., and Temel. R.E. (2014) Acute sterol O-acyltransferase (SOAT2) knockdown rapidly mobilizes hepatic cholesterol for fecal excretion. PLos One *9,* e98953.

Myers, D.S., Ivanova, P.T., Milne, S.B., and Brown, H.A. (2011). Quantitative analysis of glycerophospholipids by LC-MS: Acquisition, data handling and interpretation. Biochim. Biophys. Acta *1811,* 748-757.

Rong, X., Albert, C.J., Hong, C., Duerr, M.A., Chamberlain, B.T., Tarling, E.J., Ito, A., Gao, J., Wang, B., Edwards, P.A., et al. (2013) LXRs regulate ER stress and inflammation through dynamic modulation of membrane phospholipid composition. Cell Metab. *18,* 685-697

Smyth, G.K. (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. *3,* 3.

Temel, R.E., Lee, R.G., Kelley, K.L., Davis, M.A., Shah, R., Sawyer, J.K., Wilson, M.D., and Rudel, L.L. (2005) Intestinal cholesterol absorption is substantially reduced in mice deficient in both ABCA1 and ACAT2. J. Lipid Res. *46,* 2423-2431.

Turley, S.D., and Dietschy, J.M. (1978) Re-evaluation of the 3 alpha-hydroxysteroid dehydrogenase assay for total bile acids in bile. J. Lipid Res. *19,* 924-928.

Wang, Z., Klipfell, E., Bennett, B.J., Koeth, R., Levison, B.S., Dugar, B., Feldstein, A.E., Britt, E.B., Fu, X., Chung, Y.M., et al. (2011). Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature *472*, 57–63.

Table S1 (Included as Excel Spreadsheet). Differentially Expressed Genes (DEGs) in Acute and Chronic Mouse Models of TICE Stimulation, Related to Figure 1. For microarray analyses in the acute TICE model, female C57BL/6 mice were treated with a non-targeting control ASO (Con. ASO) or an ASO targeting the knockdown of ACAT2 (A2 ASO) as previously described for 1 week (Marshall et al., 2014). For Microarray analysed in the chronic TICE mouse model, female wild type (WT) or NPC1L1-liver transgenic mice ($NPC1L1^{Tg}$; Temel et al., 2010) were maintained on a high cholesterol diet (0.2%, wt/wt) for 8 weeks. Differentially expressed genes (DEGs) are shown for both array datasets, with a p-value threshold set at either $p<0.005$ or $p<0.001$ (n = 4-5).

Table S2 (Included as Excel Spreadsheet). Differentially Expressed Genes (DEGs) Shared in both Acute and Chronic Mouse Models of TICE Stimulation, Related to Figure 1. For microarray analyses in the acute TICE model, female C57BL/6 mice were treated with a non-targeting control ASO (Con. ASO) or an ASO targeting the knockdown of ACAT2 (A2 ASO) as previously described for 1 week (Marshall et al., 2014). For Microarray analysed in the chronic TICE mouse model, female wild type (WT) or NPC1L1-liver transgenic mice ($NPC1L1^{Tg}$; Temel et al., 2010) were maintained on a high cholesterol diet (0.2%, wt/wt) for 8 weeks. Differentially expressed genes (DEGs) shared in both array datasets are shown here, with a p-value threshold set at either $p<0.005$ (n = 4-5).

Table S3 (Included as Excel Spreadsheet). Complete Microarray Dataset from the Acute Mouse Model of TICE Stimulation (Control ASO vs. ACAT2 ASO), Related to Figure 1. Female C57BL/6 mice were treated with a non-targeting control ASO (Con. ASO) or an ASO targeting the knockdown of ACAT2 (A2 ASO) as previously described for 1 week (Marshall et al., 2014), and data here show all probesets represented in the resulting microarray analyses using mouse liver as an RNA source.

Table S4 (Included as Excel Spreadsheet). Complete Microarray Dataset from the Chronic Mouse Model of TICE Stimulation (WT vs NPC1L1-Liver-Transgenic), Related to Figure 1. Female wild type (WT) or NPC1L1-liver transgenic mice ($NPC1L1^{Tg}$; Temel et al., 2010) were maintained on a high cholesterol diet (0.2%, wt/wt) for 8 weeks, and data here show all probesets represented in the resulting microarray analyses using mouse liver as an RNA source.

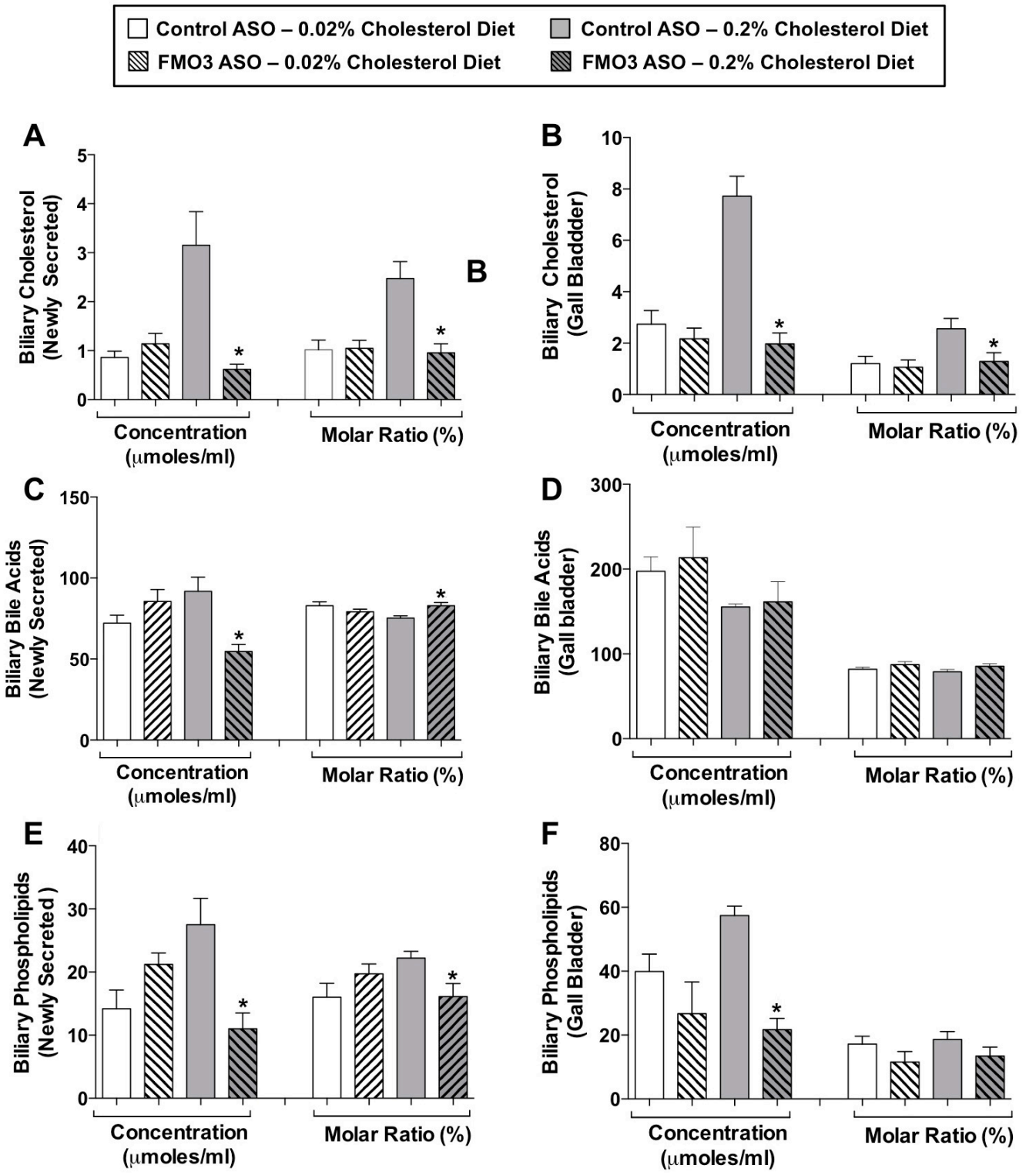

Figure S1. FMO3 Reorganizes Biliary Lipid Secretion, Related to Figure 2.

Female C57BL/6 mice were fed either a low (0.02%, wt/wt) or high (0.2%, wt/wt) cholesterol diet and treated with either a control (non-targeting) ASO or an ASO targeting knockdown of FMO3 for 6 weeks. Bile was collected from the gall bladder in one set of mice (Panels B, D, and F), and in another set of mice the common bile duct was cannulated to collect newly secreted hepatic bile (Panels A, C, and E).

(A and B) Biliary cholesterol levels.

(C and D) Biliary bile acid levels.

(E and F) Biliary phospholipid levels.

Data represent the mean ± S.E.M. from 4-10 mice per group. * = significantly different that the control ASO group within each diet group ($p<0.05$).

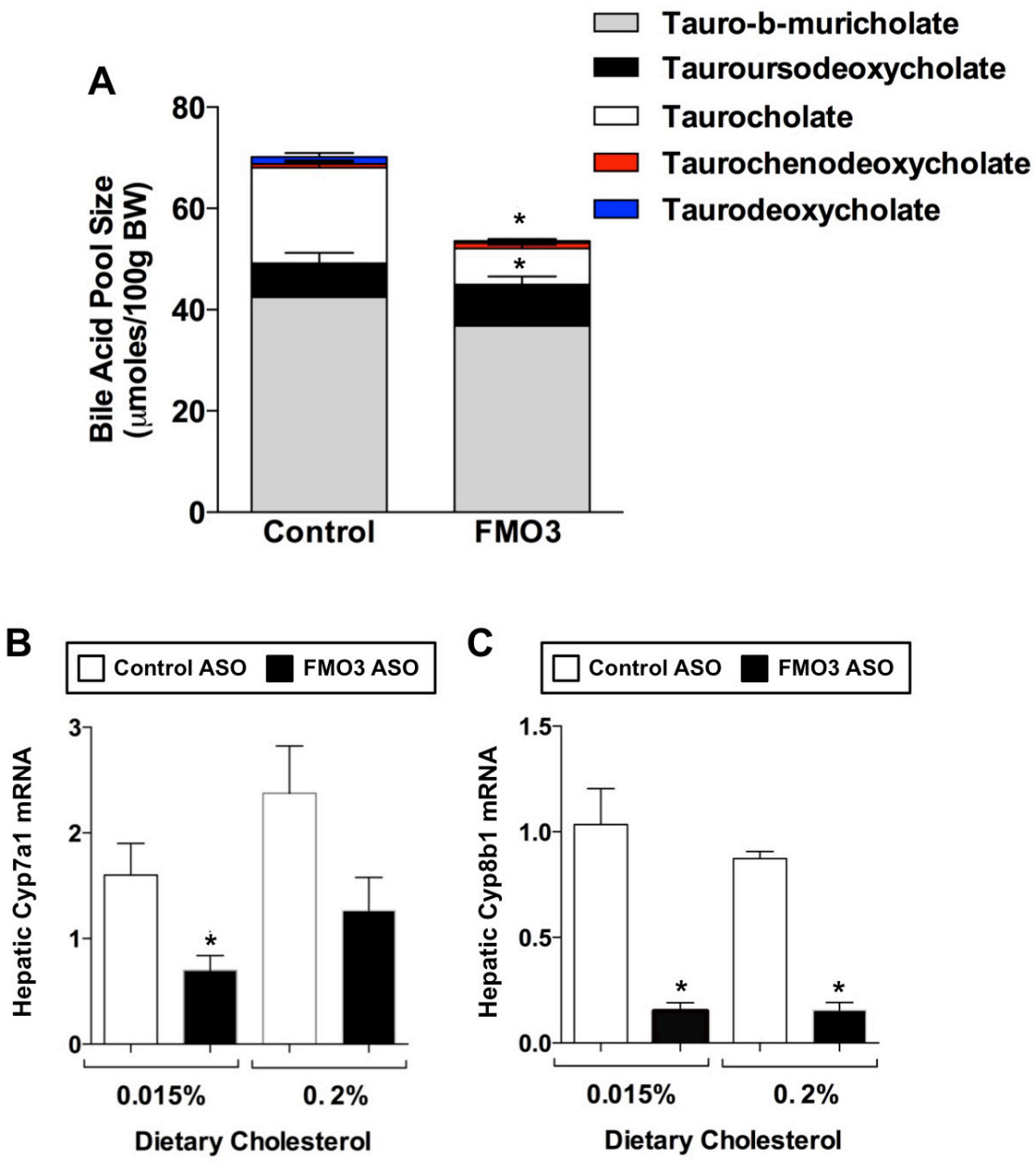

Figure S2. FMO3 Inhibition Reduces Bile Acid Pool Size, Related to Figure 2 and Figure 4.

Female C57BL/6 mice were fed a high (0.2%, wt/wt) cholesterol diet and treated with either a control (non-targeting) ASO or an ASO targeting knockdown of FMO3 for 6 weeks. Thereafter, the total bile acid pool size was determined and associated gene expression examined.

(A) Bile acid pool size and composition is altered by FMO3 inhibition.

(B) qPCR quantification of Cyp7a1 mRNA levels in the liver.

(C) qPCR quantification of Cyp8b1 mRNA levels in the liver.

Data represent the mean ± S.E.M. from 5 mice per group. * = significantly different that the control ASO group within each time

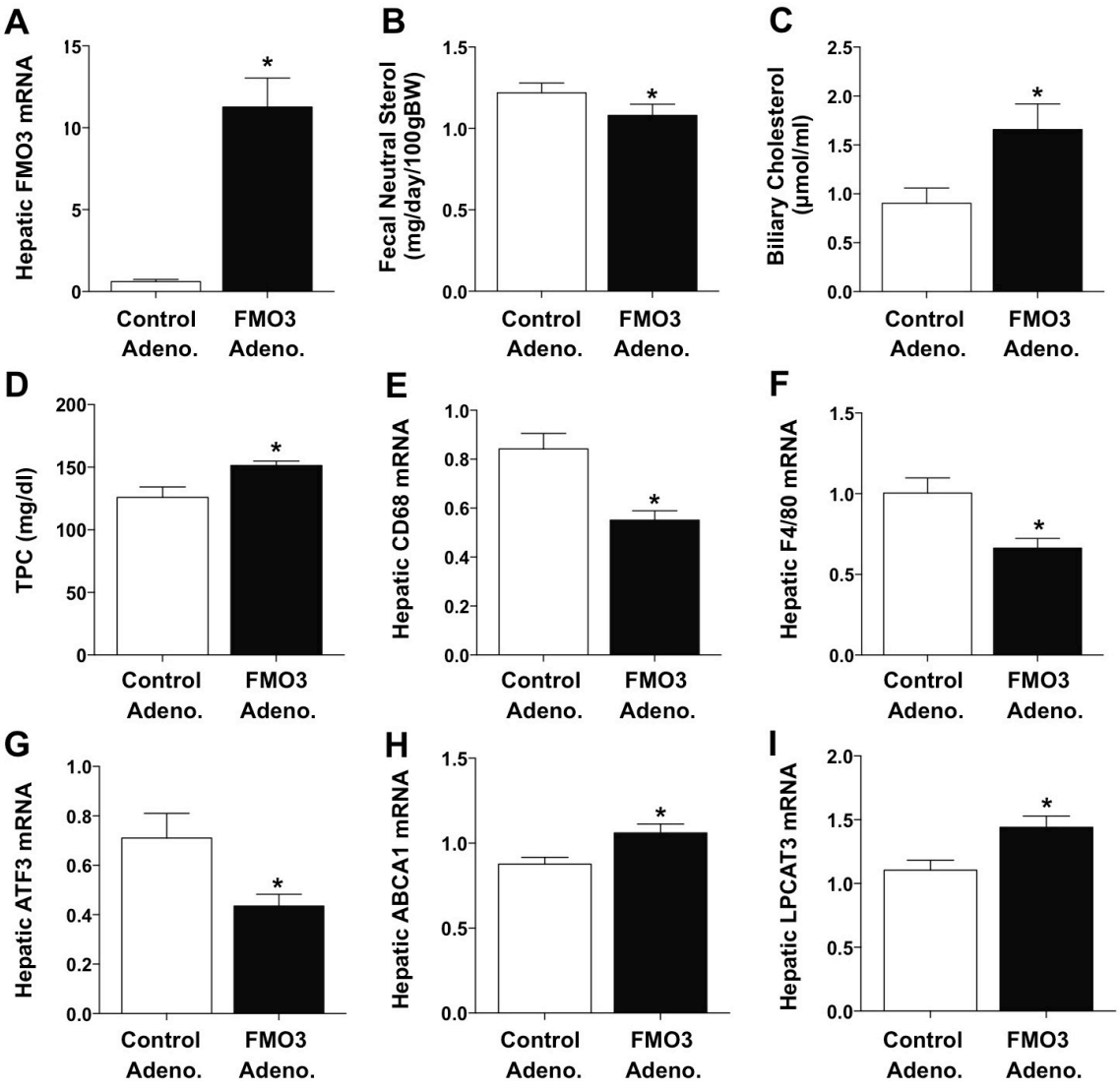

Figure S3. FMO3 Gain of Function Reorganizes Cholesterol Balance and Dampens Hepatic Inflammation and ER Stress, Related to Figures 2-5.

Male C57BL/6 mice were fed a low (0.02%, wt/wt) cholesterol diet for a total of 3 weeks. Following two weeks of dietary induction, mice received intravenous injection of either a control adenovirus (Control Adeno.) or an adenovirus driving overexpression of murine FMO3 (FMO3 Adeno.). Plasma was collected 3 days post adenoviral administration. Tissues and gall bladder bile were collected 7 days post adenoviral administration. Feces were quantitatively collected during days 4-7 post adenoviral administration.

(A) qPCR quantification of FMO3 mRNA levels in the liver.

(B) Fecal neutral sterol excretion was determined by gas liquid chromatography.

(C) Cholesterol levels in gall bladder bile.

(D) Total plasma cholesterol (TPC) levels 3 days post adenovirus administration.

(E) qPCR quantification of CD68 mRNA levels in the liver.

(F) qPCR quantification of F4/80 mRNA levels in the liver.

(G) qPCR quantification of ATF3 mRNA levels in the liver.

(H) qPCR quantification of ABCA1 mRNA levels in the liver.

(I) qPCR quantification of LPCAT3 mRNA levels in the liver.

Data represent the mean ± S.E.M. from 6-8 mice per group. * = significantly different that the control adenovirus group ($p<0.05$).

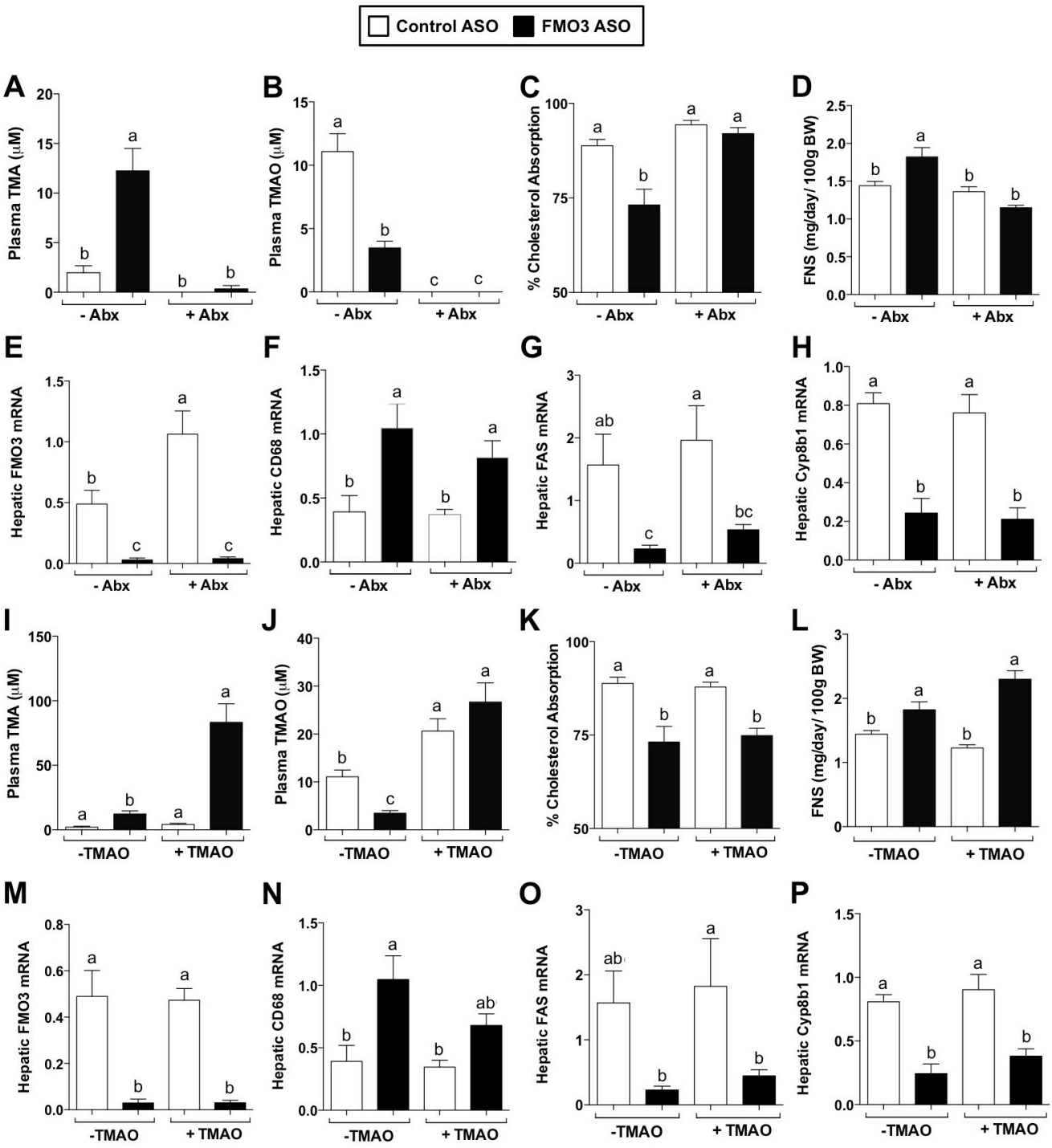

Figure S4. FMO3 Knockdown Regulates Intestinal Cholesterol Balance in a Gut Microbe-Dependent, but TMAO-Independent, Manner. **Related to Figure 2-5.** For antibiotic suppression studies (panels A-H) female C57BL/6 mice were started on a low (0.02%, wt/wt) cholesterol diet at 6-8 weeks of age and injected intraperitoneally with either a control ASO or FMO3 ASO for a total of 5 weeks. Following four weeks of dietary induction and ASO treatment, mice either continued to receive normal drinking water or were switched to water supplemented with a poorly absorbed antibiotic cocktail as previously described (Wang et al., 2011) for 7 consecutive days. For dietary TMAO add back studies (panels I-P) female C57BL/6 mice were started on a low (0.02%, wt/wt) cholesterol diet at 6-8 weeks of age and injected intraperitoneally with either a control ASO or FMO3 ASO (50 mg/kg body weight per week) for a total of 5 weeks. Following four weeks of dietary induction and ASO treatment, mice either continued to receive the same (0.02%, wt/wt) cholesterol diet or were switched to a diet containing 0.02%, wt/wt cholesterol with supplemental TMAO (0.3% wt/wt) for 7 consecutive days.

(A & I) Circulating levels of the FMO3 substrate trimethylamine (TMA).

(B & J) Circulating levels of the FMO3 product trimethylamine-N-oxide (TMAO).

(C & K) Fractional cholesterol absorption was determined using the dual fecal isotope method.

(D & L) Fecal neutral sterol excretion was determined by gas liquid chromatography.

(E & M) qPCR quantification of hepatic FMO3 mRNA levels.

(F & N) qPCR quantification of hepatic CD68 mRNA levels.

(G & O) qPCR quantification of hepatic FAS mRNA levels.

(H & P) qPCR quantification of hepatic Cyp8b1 mRNA levels.

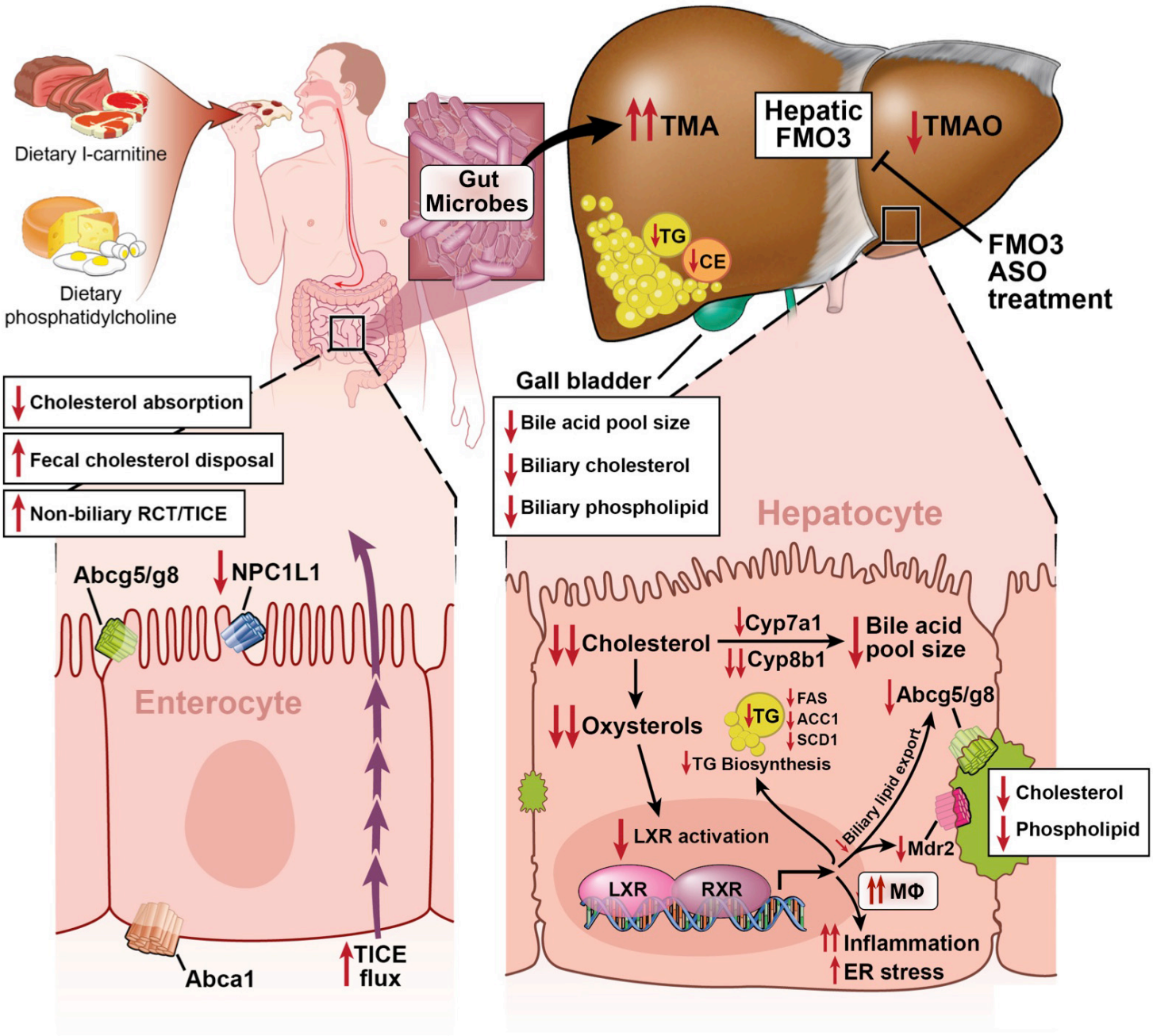

Figure S5. Working Model Summarizing Mechanisms by Which FMO3 Knockdown Impacts Whole Body Cholesterol Balance, Related to Figures 1-5.

Shown is the reorganization of cholesterol balance in the liver and small intestine in FMO3 ASO treated mice. Abbreviations: ABCA1, ATP-binding cassette transporter A1; Abcg5/g8, ATP-binding cassette transporters g5 and g8; ASO, antisense oligonucleotide; ACC1, acetyl-CoA carboxylase 1; antisense oligonucleotide; CE, cholesteryl ester; Cyp7a1, cytochrome P450 – family 7 – subfamily B – polypeptide 1; Cyp8b1, cytochrome P450 – family 8 – subfamily B – polypeptide 1; FAS, fatty acid synthase; FMO3, flavin monooxygenase 3; LPCAT3, lysophosphatidylcholine acyltransferase 3; LXR, liver X receptor; MΦ, macrophage; Mdr2, multidrug resistance protein 2; NPC1L1, Niemann Pick C1-Like 1; PL, phospholipid; RXR, retinoid X receptor; SCD1, steroyl-CoA desaturase 1; TICE, transintestinal cholesterol excretion, TMA, trimethylamine; TMAO, trimethylamine-N-oxide; TG, triglyceride.